scriptional switch depends on the ordered recruitment of GCN5/PCAF and CBP, which acetylate HMGI(Y) at distinct lysine residues, inducing opposite effects on enhanceosome stability. It takes approximately 4 hours after viral infection to assemble the enhanceosome and 2 more hours to synthesize the first IFN-β transcripts. We speculate that each of the transcription factors associates with the enhancer only weakly at the onset of viral infection, despite the presence of HMGI(Y), because it is not acetylated by GCN5/PCAF. Then, GCN5/PCAF is recruited and acetylates histones and HMGI(Y) at K71, thus "locking" the enhanceosome into a "metastable" configuration that initiates subsequent steps of the recruitment program, such as CBP-PolII, SWI/SNF, and TFIID recruitment (*4*). Thus, at the time of peak HMGI(Y) K71 acetylation (5 hours after infection), all IFN-β activators are found on the enhancer at their highest amounts, indicating stable enhanceosome assembly. However, CBP recruited to the enhanceosome cannot acetylate HMGI(Y) at K65, because HMGI(Y) is already acetylated at K71 by GCN5/PCAF. K65 acetylation and subsequent enhanceosome disassembly correlate with K71 deacetylation. Thus, the ordered and highly controlled acetylation of HMGI(Y) by two distinct HAT coactivators coordinates the IFN-β transcriptional switch by instructing either enhanceosome assembly or disassembly.

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11. HMGI(Y) derivatives were acetylated in vitro as previously described (*8*) with the following modifications. Substrates were incubated with enzyme and ^{3}H-labeled acetyl CoA (Sigma, St. Louis, MO) for 1 hour, and fresh enzyme was added every hour for 4 hours. Using ^{3}H-labeled HMGI(Y) proteins, GST pull-down assays were performed essentially as described (*3*). For peptide competition assays, the indicated amount of peptide (obtained from Research Genetics, Huntsville, AL) was added during incubation of GST-p50 with in vitro translated ^{35}S-labeled HMGI(Y) (TnT in vitro translation kit; Promega, Madison, WI).
12. DNase I footprinting experiments were carried out as described (*2*). For reactions containing preacetylated HMGI(Y), acetylation was carried out as described above (*11*) except that cold acetyl-CoA (Pharmacia, Peapack, NJ) was used in these reactions. HMGI(Y) was acetylated in situ (Fig. 3) according to methods in (*8*).
13. Chromatin IP experiments were carried out as described (*4*).
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15. Acetylated peptides were synthesized for K65 (Genosys, The Woodlands, TX) and K71 (Research Genetics) in order to immunize rabbits. Whole rabbit serum was purified by passing it over a column conjugated to the acetylated peptide and then passing the eluant over a column conjugated to the unacetylated peptide in order to deplete nonspecific antibodies. The flow-through was collected and used in Western blots and chromatin IP experiments (Fig. 2, B and C).
16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Cytokine-Specific Transcriptional Regulation Through an IL-5Rα Interacting Protein

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Cytokine receptors consist of multiple subunits, which are often shared between different receptors, resulting in the functional redundancy sometimes observed between cytokines. The interleukin 5 (IL-5) receptor consists of an IL-5–specific α-subunit (IL-5Rα) and a signal-transducing β-subunit (βc) shared with the IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors. In this study, we sought to find a role for the cytoplasmic domain of IL-5Rα. We show that syntenin, a protein containing PSD-95/Discs large/zO-1 (PDZ) domains, associates with the cytoplasmic tail of the IL-5Rα. Syntenin was found to directly associate with the transcription factor Sox4. Association of syntenin with IL-5Rα was required for IL-5–mediated activation of Sox4. These studies identify a mechanism of transcriptional activation by cytokine-specific receptor subunits.

The IL-5Rα is expressed on B cells and on eosinophilic and basophilic granulocytes (*1–3*), and IL-5 mediates the differentiation and survival of eosinophils (*4*). Furthermore, IL-5 induces the proliferation of progenitors of B1 cells and induces differentiation of mature B1 cells and conventional B2 cells into immunoglobulin-producing cells (*5*, *6*). We reasoned that if the specific α chains of cytokine receptors play a role in signaling, this may be mediated by α chain–associated proteins. However, the identity of such proteins has remained elusive. To identify proteins specifically mediating IL-5Rα signaling, we performed a two-hybrid screen using the IL-5Rα cytoplasmic domain. We constructed a two-hybrid cDNA library from human granulocytes known to express the IL-5Rα (*7*). Syntenin, protein containing tandem PDZ domains, associated specifically with the cytoplasmic domain of IL-5Rα in yeast (Fig. 1A). In glutathione *S*-transferase (GST) co-precipitation (pull-down) analyses (*8*), IL-5Rα associated with syntenin in vitro (Fig. 1B). We tested whether the association between syntenin and IL-5Rα was direct with the use of BIAcore surface plasmon resonance technology. Syntenin associated with immobilized GST–IL-5Rα$_{cyt}$ fusion protein with a calculated dissociation constant (K_d) value of 470 nM (Fig. 1C, upper panel). No binding was observed between syntenin and immobilized GST (Fig. 1C, lower panel). Syntenin, therefore, associates directly with IL-5Rα$_{cyt}$, and the binding affinity observed is similar to that obtained for the interaction of isolated PDZ domains with optimized peptides containing consensus PDZ-binding motifs (*9*). Syntenin co-immunoprecipitated with IL-5Rα from mammalian cells, indicating that the protein association can occur in vivo (Fig. 1D). Syntenin did not bind to GST fusion proteins derived from either the IL-3 or the GM-CSF receptor (*10*).

Deletion mutants of IL-5Rα were then used to identify the region of interaction with syntenin. Deletion of the last 15 carboxyl-terminal residues abolished the association with syntenin (Fig. 2A). A comparison of the amino acid sequences between mouse and human IL-5Rα revealed that although these sequences were somewhat variant, two of the four COOH-ter-

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minal residues (human, DSVF; mouse, NSTF) (*11*) were conserved. Syntenin contains two tandem PDZ domains, and these domains are known to interact with COOH-terminal peptide sequences (*12*). A single-point mutant of IL-5Rα in which the COOH-terminal phenylalanine was substituted for an alanine residue (IL-5Rα F →A) completely abrogated the interaction between syntenin and IL-5Rα (Fig. 2B). Single point mutation of other amino acids in the COOH terminus of IL-5Rα_{cyt} did not affect syntenin binding (*13*). Using different deletion mutants of syntenin, we investigated which region of syntenin was responsible for the association with IL-5Rα. Deletion of either of the PDZ domains completely abrogated the IL-5Rα–syntenin interaction (Fig. 2C).

In a second two-hybrid screen, syntenin was identified as a binding partner of the transcriptional activator Sox4 (Fig. 1A). Sox4 is a member of a large family of transcription factors that share homology in their DNA binding domain, the so-called high mobility group (HMG)-box (*14*, *15*). Sox4 associated specifically with syntenin (Fig. 1A), and no association was found between Sox4 and IL-5Rα (Fig. 1A) or between syntenin and the closest relative of Sox4, Sox11 (*13*). GST pull-down analysis of Sox4 using different deletion mutants of syntenin revealed that Sox4 was able to bind to syntenin when both PDZ domains had been removed (Fig. 3A). This association was also found in co-immunoprecipitation assays in mammalian cells (Fig. 3B) (*16*).

Because both IL-5 and Sox4 have been implicated in B cell development (*17*–*20*), we tested whether Sox4 itself could be activated by IL-5. As a relevant model system, we used the cytokine-dependent early mouse pre-B cell line BaF3, endogenously expressing syntenin and stably transfected with IL-5Rα (*21*). IL-5 induced the activation of a luciferase reporter bearing seven copies of a Sox4 DNA-binding motif (AACAAAG) (*14*) (Fig. 3C, left panel). A second reporter bearing seven copies of a mutated motif (CCGCGGT) was not activated, demonstrating Sox4-specificity (Fig. 3C, right panel). Analyses of syntenin and Sox4 expression after IL-5 stimulation revealed that both exhibited cytoplasmic and nuclear staining (*10*). IL-3 was unable to induce activation of the luciferase reporter in BaF3 cells (Fig. 3C).

To investigate whether syntenin is critical for IL-5–mediated activation of Sox4, we measured the Sox4 reporter activation after transfection of increasing amounts of antisense syntenin, which led to a dose-dependent decrease in Sox4 activation (Fig. 4A, left panel). As a control, we measured IL-5–induced activation of a cyclin D1 reporter construct that was unaffected by antisense syntenin (Fig. 4A, right panel). IL-5–stimulated ERK-1 phosphorylation was also unaltered, demonstrating that receptor-mediated signaling pathways are not aspecifically abrogated (*10*). We next tested the ability of the COOH-terminal deletion mutants of IL-5Rα to activate Sox4. Deletion of the COOH terminus of IL-5Rα or the COOH-terminal point mutant IL-5Rα (F → A) resulted in loss of Sox4 activation (Fig. 4B), whereas ERK phosphorylation by these receptors was unaffected (Fig. 4C). Thus, the ability of syntenin to associate with IL-5Rα also appears to be critical for IL-5–mediated Sox4 activation.

These data demonstrate a mechanism of transcriptional activation by cytokine-specific receptor subunits. Sox4 activation by IL-5Rα appears to be direct, with syntenin functioning as an adaptor molecule. An analogy emerges with the activation of two families of

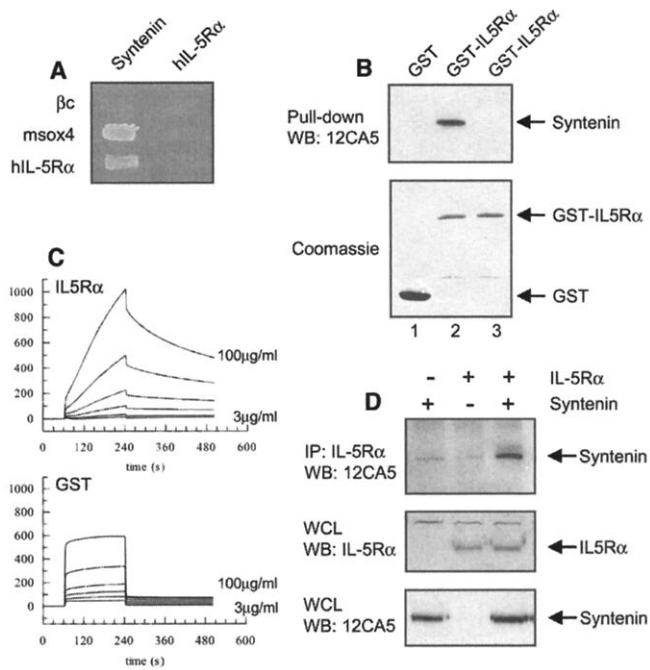


Fig. 1. Syntenin associates specifically and directly with IL-5Rα. (**A**) *S. cerevisiae* strain HF7C was transformed with constructs expressing the GAL4 DNA binding domain fused with βc, Sox4, or IL-5Rα in combination with constructs expressing the transactivation domain of GAL4 fused to either human syntenin or IL-5Rα as indicated. Yeast was grown on selective medium (-Trp, -His, -Leu) for four days. (**B**) COS cells were transiently transfected with expression constructs for either HA-syntenin (lanes 1 and 2) or empty vector (lane 3). GST pull-down assays were performed on the cell lysates using either GST (lane 1) or GST–hIL-5Rα (lanes 2 and 3). The associated syntenin was visualized by Western blotting (WB) using antibody to epitope (upper panel). The blot was stained with Coomassie to show equal amounts of protein in each lane (lower panel). (**C**) GST–IL-5Rα (upper panel) fusion protein or GST alone (lower panel) was immobilized onto a CM5 Biosensor chip using standard techniques. Binding of full-length GST-syntenin fusion protein was detected by surface plasmon resonance. Protein was injected at concentrations of 100, 50, 25, 12.5, 6.25, and 3.13 μg/ml, respectively. (**D**) COS cells were transiently transfected with expression constructs for HA-syntenin (lanes 1 and 3) and hIL-5Rα (lanes 2 and 3). IL-5Rα was immunoprecipitated (IP) and the associated syntenin was visualized using antibody to epitope (upper panel). Five percent of the cell lysate was used for SDS-PAGE followed by Western blotting using antibody to IL-5Rα (middle panel), and syntenin expression was verified using antibody to epitope (lower panel).

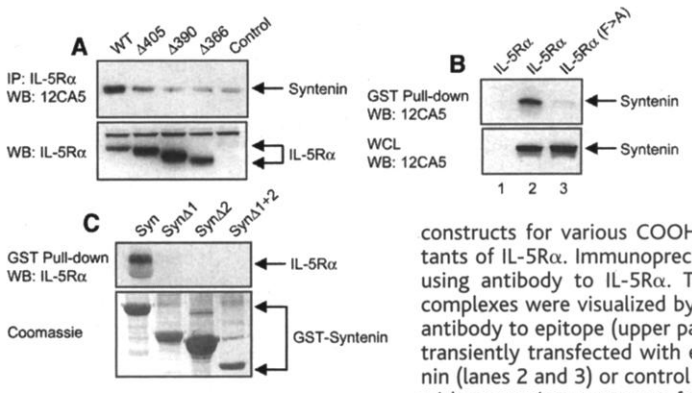


Fig. 2. Syntenin-PDZ domains mediate the association with the carboxy terminus of IL-5Rα. (**A**) COS cells were transfected with HA-tagged syntenin and either control vector or expression constructs for various COOH-terminal deletion mutants of IL-5Rα. Immunoprecipitation was performed using antibody to IL-5Rα. The immunoprecipitated complexes were visualized by Western analysis using antibody to epitope (upper panel). (**B**) COS cells were transiently transfected with either HA-tagged syntenin (lanes 2 and 3) or control vector (lane 1) together with expression constructs for IL-5Rα (lanes 1 and 2) or a point mutant IL-5Rα (F → A) where the COOH-terminal phenylalanine was mutated to alanine (lane 3). Immunoprecipitation was performed on the cell lysates using a rabbit polyclonal antibody to IL-5Rα. The immunoprecipitated complexes were visualized by Western analysis using antibody to epitope (upper panel). (**C**) COS cells were transiently transfected with expression constructs for IL-5Rα. GST pull-down analysis was performed on the cell lysates using GST-syntenin fusion proteins. The associated IL-5Rα was visualized by Western blotting using antibody to IL-5Rα (upper panel).

transcription factors, the signal transducers and activators of transcription (STAT) and particularly the SMAD family. STAT transcription factors associate directly with phosphorylated tyrosine residues in the receptor (*22*). The SMAD transcription factors Smad2 and Smad3 associate with the transforming growth factor–β (TGF-β)/activin receptors, and in the "silent" state this complex is stabilized by the membrane-bound adaptor molecule Smad Anchor for Receptor Activation (SARA). SARA then dissociates from the SMADs upon their activation by the receptor, releasing the activated SMAD (*22*). Because syntenin is also reported to associate with other membrane receptors including syndecans (*23*) and ephrins (*24*), a more widespread role in transcriptional regulation might be possible.

Functional dissection of the IL-5Rα chain has revealed that carboxy-terminal regions of this receptor are important for regulating B cell differentiation (*25*). Sox4(−/−) mice exhibit a defect in B cell development (*17*), whereas IL-5Rα(−/−) mice show impaired development of a subpopulation of B cells, the B1 cells (*18–20*). Taken together, these studies suggest that IL-5Rα–induced Sox4 activation could play a role in the regulation of early B cell development. The importance of this signaling pathway in IL-5–mediated regulation of hematopoietic cells remains to be elucidated.

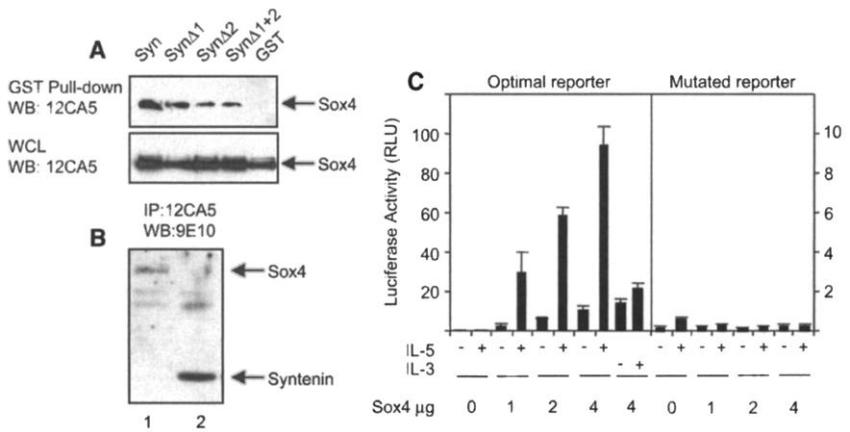
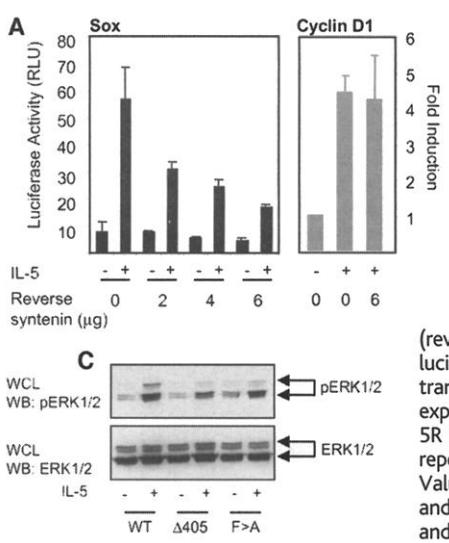


Fig. 3. Sox4 associates with syntenin and is activated by IL-5. **(A)** COS cells were transiently transfected with expression constructs for HA-Sox4. GST pull-down analysis was performed on the cell lysates using GST-syntenin fusion proteins. The associated Sox4 was visualized by Western blotting using antibody to epitope (upper panel). **(B)** COS cells were transfected with either HA-syntenin and Myc-Sox4 (lane 1) or Myc-syntenin and HA-Sox4 (lane 2). After anti-HA immunoprecipitation with 12CA5, samples were analyzed by anti-Myc Western blotting (9E10). **(C)** BaF3–IL-5Rα cells were transfected with either luciferase reporter construct containing a mSox4 transcriptional element or a mutant thereof, together with increasing amounts of Sox4, before being treated as indicated. Values shown represent the relative luciferase activity and are corrected for variations in transfection efficiency and growth.



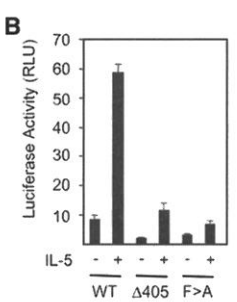


Fig. 4. Syntenin mediates IL-5–induced Sox4 activation. **(A)** BaF3–IL-5Rα cells were transfected either with Sox4 and the appropriate Sox luciferase reporter construct (left panel) or with a cyclin D1 luciferase reporter construct (right panel), together with increasing amounts of antisense (reverse) syntenin. Values shown represent the relative luciferase activity and are corrected for variations in transfection efficiency and growth. **(B)** BaF3 cells stably expressing hIL-5Rα (WT), hIL-5Rα Δ405 (Δ405), or hIL-5R F → A(F > A) were transfected with Sox4 luciferase reporter construct and an expression construct for Sox4. Values shown represent the relative luciferase activity and are corrected for variations in transfection efficiency and growth. **(C)** The cell lines used in (B) were serum-starved for 4 hours. Cells were then stimulated with buffer (–) or recombinant IL-5 (+) for 15 min before lysis in boiling sample buffer. Western blotting was performed, and phosphorylated ERK1 and ERK2 were visualized using antibody to phospho-ERK (upper panel). The blot was stripped and re-probed using antibody to ERK1 and -2 (lower panel).

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7. The intracellular domain of human IL-5Rα (hIL-5Rα) and the box1 region of human βc were cloned in frame with the Gal4-DNA binding domain in pGBT8. Sox4 was cloned in frame with the Gal4-DNA binding domain in pMD4. pGBT8–IL-5Rα was used to screen a two-hybrid library generated from granulocyte-cDNA (30% eosinophils and 70% neutrophils) with the use of the Stratagene λ-ZAP kit (Amsterdam, The Netherlands). pMD4-mSox4 was used to screen a human fetal liver library (HL4029AH; Clontech, Woerden, The Netherlands). Human syntenin and the intracellular domain of hIL-5Rα were cloned in frame with the Gal4 transactivation domain in pGADGH (Clontech). Plasmids were transformed into the *Saccharomyces cerevisiae* strain HF7C (Clontech).
8. COS cells were transiently transfected with expression constructs as indicated by the calcium phosphate precipitation method. Immunoprecipitation of protein complexes using antibody to HA epitope, 12CA5 (Boehringer, Almere, The Netherlands) or rabbit polyclonal antibody to IL-5Rα was performed using standard techniques. For fusion protein-binding assays, GST fusion proteins were purified from isopropyl-β-D-thiogalactopyranoside (IPTG)–induced *Escherichia coli* BL21 cultures. Approximately 10 μg of GST protein was pre-coupled to glutathione agarose beads, which were used to precipitate protein complexes from transiently transfected COS cells using standard techniques.
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21. BaF3 cells stably expressing hIL-5Rα (BaF3–IL-5Rα) were electroporated with a luciferase reporter plasmid containing seven optimal Sox4 sites or its negative control containing mutated sites, the internal transfection control (50-ng pRL-TK; Promega, Leiden, The Netherlands), and Sox4 and reverse syntenin expression constructs as indicated. After transfection, cells were cultured either with or without recombinant IL-5 (10^{-10} M). After 12 hours, cells were harvested and luciferase activity was measured. Values were corrected for transfection efficiency and growth and represent the mean of at least three independent experiments (± SEM).
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